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# Method for identifying nucleic acids by means of matrix-assisted laser desorption/ionization mass spectrometry

The present invention relates to a method for the detection of a nucleotide sequence in a nucleic acid molecule by means of pre-determined probes with different mass by means of matrix-assisted laser desorption/ionization mass spectrometry. One advantage of the method of the invention is that it allows for the simultaneous characterization of a variety of unknown nucleic acid molecules by using a set of various probes. Furthermore, this invention relates to a kit containing the probes and/or a probe support, optionally with the nucleic acid molecules linked thereto.

The exact characterization of nucleic acids is very complex and expensive. Unknown DNA can be characterized by sequencing. That is the most precise way of analyzing DNA. Sequencing DNA, however, requires a lot of time and effort and is necessary only if the whole sequence is of interest. Only very short DNA parts (<1000 nucleobases) can be sequenced in one working process. If DNA fragments longer than 1000 nucleobases are to be analyzed on a large scale, it is necessary to subdivide the DNA, which makes the process more expensive.

Many statements can be made, however, even with a lower resolution. The methods described so far, however, are disadvantageous in so far as radioactivity might possibly have to be used and only one single probe can be used in an analysis. Such a method from the state of the art comprises, for example, a search for some information by means of an array of various target DNAs. An array with many thousand target DNAs can be immobilized on a solid phase, and subsequently all the target DNAs can be examined together with view to the presence of a sequence by means of a probe (nucleic acid with a complementary sequence).[1,2] A match of the target DNA with the probe can be proven by hybridization of both nucleic acids. Probes can be any nucleic acid sequences of different lengths. There are various methods for the selection of ideal libraries of probe sequences which overlap minimally.[3,4] Probe sequences can be arranged specifically in order to find specific

target DNA sequences. One approach where this technology is made use of is oligofingerprinting. A library of target DNAs is scanned with short nucleic acid probes. Usually the probes for that have a length of only 8-12 bases. One probe is hybridized all at once with a target DNA library which has been immobilized on a nylon membrane. The probe is radioactively labelled and the hybridization is estimated according to the localisation of the radioactivity. For scanning an immobilized DNA array fluorescently marked probes have also been used.[5] A similar method is used for multiplexing the sequencing of DNA.[6,7] Various vector systems are used for cloning target sequences. One clone each is pooled by each cloning vector, the sequence reaction is carried out, the fragments are separated on the gel and the gel is blotted on to a nylon membrane. The various sequences of the cloning system are subsequently hybridized with the immobilized DNA so that the sequence belonging to the respective cloning system is obtained. Hereby, the scanning of the cloning system can also be carried out by means of a probe which is detectable by mass spectrometry.[8]

Any molecules capable of interacting sequence-specifically with a target DNA can be probes. Most commonly used are the oligodeoxyribo nucleotides. For this purpose, however, any modification of nucleic acids, such as peptide nucleic acids (PNA)[9,10], phosphorothioate oligonucleotide or methyl phosphonate oligonucleotide, is suitable. The specificity of a probe is extremely important. Phosphorothioate oligonucleotides are not particularly preferred as their structure is modified by the sulphur atoms, which has a negative influence on the properties of the hybridization. This can be due to the fact that usually phosphorothioate oligonucleotides are not synthesized free of diastereomers. In the past, there has been a similar purity problem with methyl phosphonat oligonucleotides, but these oligonucleotides are increasingly synthesized free of diastereomers. An essential difference between methyl phosphonate oligonucleotides and phosphorothioate oligonucleotides is the uncharged backbone of the former which leads to a reduced hybridization dependency on buffer salts and, on the whole, to a higher affinity due to reduced rejection. Peptide nucleic acids also have uncharged backbones which, at the same time, drastically deviate in their chemical properties from the common sugar phosphate structure of the backbone of nucleic acids. The backbone of a PNA exhibits an amide sequence instead of a sugar phosphate backbone of normal DNA. PNA hybridizes very well with a sequence which is complementary to DNA. The melting temperature of a PNA/DNA hybrid is higher than the one of the corresponding DNA/DNA hybrid and, again, the hybridization dependency on buffer salts is relatively low.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) is a novel, very efficient method for the analysis of biomolecules.[11] An analyte molecule is imbedded in a light-absorbing matrix. The matrix is steamed off by a short laser pulse and the analyte is thus transferred into the gas phase in an unfragmented form. The ionization of the analyte is achieved by the pulses of the matrix molecules. An applied voltage accelerates the iones in a zero-field flight pipe. Due to their differing mass the iones are accelerated differently. Small iones reach the detector earlier than big ones. The duration of the flight is converted into the mass of the iones. Technical hardware novelties have improved the method significantly. Here, delayed extraction (DE)[12] is worth mentioning. For DE the acceleration voltage is switched on with a delay after the laser pulse and, thereby, an improved resolution of the signals since the number of pulses is reduced. MALDI is perfectly suitable for the analysis of peptides and proteins. The analysis of nucleic acids is more difficult.[13] The sensitivity for nucleic acids is about 100 times worse than for peptides and decreases with increasing fragment size to a larger than proportional extent. The reason for this is that only one single proton has to be caught for the ionization of peptides and proteins. For nucleic acids which have a multifold negatively charged backbone the ionization process by means of the matrix is by far more inefficient. For MALDI the choice of the matrix plays an extremely important role. For the desorption of peptides some very efficient matrices have been found which result in very fine cristallisations. For DNA several suitable matrices have been found by now, but the difference in sensitivity could not be reduced in this way. The difference in sensitivity can be reduced by modifying the DNA chemically in such a way that it becomes similar to a peptide. Phosphorothioate oligonucleotides, wherein the normal phophate of the backbone has been substituted by thiophophates, can be converted into a charge-neutral DNA by simple alkylation chemistry.[14] The coupling of a "charge tag" to this modified DNA results in an increase of the sensitivity to the same level as it is found for peptides.[15,16] Due to this modifications the possibility arised to use similar matrices as used for the desorption of peptides. A further advantage of charge tagging is the increased resistance of the analysis against impurities which make the detection of unmodified substrates much more difficult. PNAs and methyl phosphonat oligonucleotides have been examined in MALDI and can be analysed in this way.[17,18,19]

Combinatorial syntheses,[20] i.e. the production of substance libraries starting from a mixture of precursors, are carried out both in solid and liquid phases. In particular, the combinatorial solid phase synthase has established itself early since in this case the separation of side products is particularly easy. Only the target compounds linked to the carrier (support) are retained in a washing step and isolated at the end of the

synthesis by specific decomposition of a linker. In a simple way, this technique allows for the simultaneous synthesis of a number of various compounds at a solid phase and, thus, for the obtaining of chemically "pure" substance libraries.[21,22,23] Therefore, the compound classes which are not synthesized in combinatorial, conventional syntheses on a solid phase either become suitable for combinatorial chemistry in a particular easy way. This is why they are widely used. This particularly applies to peptide, nucleic acid and PNA libraries.

The synthesis of peptides is accomplished by binding the first N-protected amino acid (e.g. Boc) to the support, subsequent removal of the protection and reaction of the second amino acid with the now free $NH_2$-group of the former. Non-reacted amino functions are removed in a further "capping" step of a following reaction in the next synthesis cycle. The protection group at the amino function of the second amino acid is removed and the next component can be coupled. For the synthesis of peptide libraries a mixture of amino acids is used in one or more steps. The synthesis of PNA and PNA libraries is carried out accordingly.

Nucleic acid libraries are usually obtained by solid phase synthesis with mixtures of various phophoramidite nucleosides. This can be carried out in commercially available DNA synthesizers without modifications in the synthesis protocols. Furthermore, various studies about combinatorial synthesis of PNA libraries have been published.[24,25] These studies deal with the construction of combinatorial sequences, i.e. the synthesis of PNAs in which single, specific bases in the sequence are replaced by degenerated bases and, thus, a random sequence variation is achieved. The use of mass spectrometric methods for the analysis of combinatorial libraries has been described repeatedly.[26,27,28,29]

There are various methods for immobilizing DNA. The best known method is the solid binding of a DNA, which is functionalized with biotin, to a streptavidin-coated surface.[30] The binding strength of this system corresponds to a covalent chemical bond without being one. For the covalent binding of a target DNA to a chemically prepared surface a corresponding functionality of the target DNA is required. DNA itself does not have any functionalization which is suitable for that. There are various ways to introduce a suitable functionalization into a target DNA: two easily manageable functionalizations are primary, aliphatic amines and thiols. Such amines are converted quantitatively with N-hydroxy-succinimidesters. Under suitable conditions thiols react quantitatively with alkyliodides. One difficulty is the introduction of such a functionalization in a DNA. The easiest method is the introduction of a

primer of a PCR. The described methods use 5'-modified primers ($NH_2$ and SH) and a bifunctional linker.[31,32,33]

When immobilizing on a surface its nature is of main importance. The systems described so far mainly consist of silicon or metal (magnetic beads). Another method for binding a target DNA is based on using a short recognizing sequence (e.g. 20 bases) in a target DNA for the hybridization to a surface-immobilized oligonucleotide.[34] Enzymatic variants have also been described for the introduction of chemically activated positions into a target DNA.[35] Here, a 5'-$NH_2$-functionalization is carried out on a target DNA enzymatically.

As described above, a number of methods are known in the state of the art which specifically aimed at an exact analysis of nucleic acids. These methods usually either require a lot of time and effort and/or money.

Thus, the technical problem underlying the present invention was to provide a fast and cost efficient method for the identification of target nucleic acids.

This technical problem has been solved by providing the embodiments characterized in the claims.

Thus, the present invention relates to a method for the detection of a nucleotide sequence in a nucleic acid molecule comprising the following steps:

(a) hybridization of nucleic acid molecules with a set of probes with different nucleobase sequences, whereby each probe has a mass which differs from the one of all other probes;
(b) separation of the probes which have not been hybridized;
(c) contact of the hybridized probes with a matrix which supports the desorption of the probes with a laser beam;
(d) analysis of the hybridized probes which are surrounded by the matrix on a probe support of electrical conductive material in a mass spectrometer; and
(e) determination of the nucleic acid molecules exhibiting the sequence, wherein the positions of the probes on the probe support allow for an allocation of the nucleic acid molecules hybridizing therewith.

The method of the invention combines - in an advantageous way - methods for analyzing arrays of target nucleic acids (oligofingerprinting) as well as the mass spectrometric analysis of nucleic acids and modified nucleic acids. Thereby, a number of various probes are used which allow for the detection of one or more

nucleotide sequence(s) in a nucleic acid molecule. A combination of these methods could not be carried out so far since the mass differentiation of the probes did not allow for clear conclusions with regard to the sequence and the sensitivity of the mass spectrometry analysis of nucleic acids did not correspond to the probe amounts of a oligofingerprinting experiment. The condition which enables a certain sequence to be detected is that probes are used in the method which allow for a hybridization with the target sequence. The determination of the nucleic acid molecules is accomplished by testing the hybridization with one of the probes.

It is clear to the person skilled in the art that it is not always possible to exactly determine a wanted nucleotide sequence by means of hybridization methods since - even under stringent hybridization conditions – a hybridization of a probe can possibly take place despite so-called "mismatches" (e.g. from a certain minimum length of a probe or with positioning of the mismatch(es) which can be tolerated during hybridization). With a given nucleotide sequence of a probe a complementary sequence in the nucleic acid molecule can only be determined with a certain estimation in part of the embodiments since apart from exact complementary sequences possibly also such sequences can be determined which are not exactly complementary in their sequences. Therefore, the nucleotide sequence comprises also homologous nucleotide sequences which exhibit a homology degree of more than 90%, particularly preferred of more than 95 %. The present invention comprises all the above-mentioned embodiments.

Depending on the hybridization conditions the method of the invention can be used to detect either specific nucleotide sequences or groups of nucleotide sequences which have a similar sequence. If e.g. stringent hybridization conditions are chosen, the probes used can hybridize only to the nucleotide sequences which are exactly complementary to their nucleotide base sequences. If, however, non-stringent hybridization conditions are chosen, the probes used can detect any nucleotide sequences which deviate from the nucleotide base sequences in such a way that they still allow for a hybridization under the chosen conditions. In that way, the method of the invention can also be used to detect homology, variants or alleles of a certain sequence. The person skilled in the art knows what stringent or non-stringent hybridization conditions are; cf e.g.Sambrook et al., "Molecualr Cloning, A Laboratory Manual" CSH Press, Cold Spring Harbor, 2nd ed. 1989, Hames and Higgins (eds.) "Nucleic Acid Hybridization, A Practical Approach", IRL Press, Oxford 1985. Stringent hybridization conditions are, for example, hybridization in 6 X SSC, 5 x Denhardt's Reagent, 0.5 % SDS and 100 µg/ml denaturated DNA at 65 °C and washing in 0.1 x SSC, 0.1 % SDS at 65°C. Non-stringent hybridization conditions differ from the above-mentioned conditions in so far as, for example, the hybridization and/or the

washing is carried out at a lower temperature, e.g. at 50°C and/or the amount of SSC is increased to e.g. 1 x or 2 x SSC.

The method of the invention also allows for the detection of several different sequences in a target DNA, wherein the different sequences are complementary to different probes. Ideally, e.g. when using probes with overlapping sequences, the whole nucleotide sequence of a target nucleic acid can be detected or clarified.

With the method of the invention it is possible to initially determine whether a probe has been applied to the probe support which exhibits a sequence that can be hybridized with a probe under the chosen conditions. If this is the case, the probe nucleic acid can be further examined and characterized. The nucleic acid which has not been used can be further examined by standard methods such as sequencing method, since for the method of the invention only a fraction of the probe has to be used for the analysis of the invention

The method of the invention can also be carried out more than once - simultaneously or consecutively – wherein the hybridization conditions are varied. In that way it is possible to, for example, determine in a target DNA array how many and which target DNAs exhibit a high homology degree before searching for specific sequences begins.

The allocation of a probe which has been hybridized at a certain position to the immobilized sample on the probe support is preferably carried out by means of a data processing system which allocates the respective recorded spectrum at a position of the probe support to the target DNA which is located at the same position. Preferably, the target nucleic acids are arranged on the surface or the probe support in a certain order.

As mentioned before, the probe support consists of electrical conductive material. This also includes the surface of the probe support where the hybridized probes which are surrounded by the matrix are located. The surface can differ from the probe support with regard to substance.

The surface where the probes for the mass spectrometry have to be directly or indirectly immobilized must be constituted in such a way as to be able to act as a probe support for a mass spectrometer (Figure 2). This means that it must be of electrical conductive material since a defined voltage has to be applied to achieve a stable acceleration of the ionized probe molecules. A non-conductive surface would

lead to an electrostatic charge which means that a shift of the mass would be observed due to the deviation in voltage and a mass assignment would be impossible.

Preferred embodiments of the present invention are described below and can also be seen in the examples.

In a preferred embodiment of the method of the invention nucleic acid molecules are transferred to the surface of a carrier according to step (a). Suitable carriers are e.g. "magnetic beads", plastic beads or glass beads which have been functionalized with streptavidin or with amino groups, SH groups or epoxy groups.

In a particularly preferred embodiment the surface of the carrier is the surface of the probe support consisting of electrical conductive material. This embodiment allows for the method to be carried out in a relatively uncomplicated way since the nucleic acid molecules do not have to be transferred to a probe support after hybridization has taken place.

In another particularly preferred embodiment of the method of the invention the carrier with the nucleic acid molecules located at its surface is applied onto the probe support consisting of electrical conductive material before step (c). The linking can be achieved e.g. by the matrix itself or by addition of nitrocellulose to the matrix.

In an also particularly preferred embodiment the hybridized probes are removed from the immobilized nucleic acid molecules before, after or during contact with the matrix in step (c) and applied to the probe support consisting of electrical conductive material. In this embodiment the complexes of hybridized nucleic acid molecules and probes are denatured and only the probes are applied to the probe support. A denaturation can be achieved by known methods such as e.g. alkaline or heat denaturation or by the acid matrix solution itself.

In another preferred embodiment the probe support has either a metal surface which is coated with glass or a chemical modified surface which allows for solid binding of the target DNA.

In a further preferred embodiment of the method of the invention the immobilization of the nucleic acid molecules on the surface of the probe support is carried out via a $NH_2$-, epoxy- or SH-function, by means of coating of the surface of the probe support

with a silicate or silane, via a protein-substrate, protein-protein or a protein-nucleic-acid-interaction or by means of interaction of two hydrophobe components.

If the probe support is coated e.g. with gold, coupling of the target DNA can be achieved by means of SH- or $NH_2$-functions introduced during the molecular biological preparation of the target DNA. The reverse option, i.e. to link modified DNA to functionalized gold particles, is also possible. The firm Nanoprobes Inc., Stony Brook, NY, sells, for example, gold nanoparticles linked with streptavidin or with amino functions. As mentioned earlier, another possibility is to coat the metal surface of the probe support with glass. Coupling of the target DNA which is bound to a bifunctional linker (e.g. SIAB, Pierce Chemical, Rockford, IL, USA) by means of a SH-functionalization can be achieved to the glass surface via amino-functionalization. Another variant is the immediate coating of the metal surface with trimethoxy-3-aminopropylsilane. It is possible to subsequently couple a target DNA to the aminofunction, via a bifunctional linker as above.

In a particularly preferred embodiment the protein-substrate-interaction is a biotin-streptavidin- or an antibody-antigen-linkage. MALDI probe supports are usually of metal (e.g. iron) at which neither proteins nor DNA molecules can be immobilized without further modification. One possibility of immobilizing is to coat the iron surface with gold as e.g. SH-functions can be bound to it. Bifunctional linkers are suitable for coupling which have a SH-function and another function which corresponds to the functionalization of the target DNA. If, e.g. the target DNA is biotin-functionalized, the linker should be coupled with streptavidin. If the target DNA is $NH_2$-functionalized, the linker can have an N-hydroxysuccinimidylester-function.

In another particularly preferred embodiment the protein-nucleic-acid-interaction is a linking of the nucleic acid to Gene32, a protein binding single DNA in an unspecific way.

In another preferred embodiment of the method of the invention the used probes are nucleic acids having a mass tag. According to this embodiment the probes can also have several tags which are located at different positions, e.g. at the 5' and the 3' end. Due to the combination of number and localization of mass tags, or as the case may be, in combination with charge tags, the versatility and the sensitivity of the method of the invention can be significantly increased.

In a particularly preferred embodiment the mass tags are also charge tags whereas the nucleic acids additionally have a charge tag in another particularly preferred embodiment.

Charge tagging can be carried out according to the method of Gut et al.[15,16] An amino-functionalized substrate (1mM) is added to trimethylamin/$CO_2$-buffer (pH = 8.5, 200 mM) on ice at 0°C with 1% ω-trimethylammoniumhexanacid-H-hydroxy-succinimidylester (CT). After 30 minutes the volatile buffer and the solvent are removed in vacuum. The amino-functionlized substrate can e.g. be a combinatorial produced library with different probes differing in mass. The masses of the substrate library can be changed by a defined amount (Figure 3) by varying the length and the functionalization of the CT. Since during the combinatorial synthesis e.g. a probe library of 64 probes with different masses (Fig.4) within a mass range of 200 Da is produced, the mass/charge tags increase the mass in units of 200 Da each. That means that the first combinatorial synthesis preparation is produced with the smallest possible charge tag, the second with a mass/charge tag which is 200 Da heavier and the third with a mass/charge tag which is another 200 Da heavier and so on. In theory, the range can be increased at will as long as the used mass spectrometer is capable of eliminating the difference between the two neighbouring probes and as long as the synthesis seems practically feasible. For probes with 10 nucleic bases a basic mass in the range of 2600-2800 Da is achieved. The used mass range with sufficient mass accuracy is below 4000 Da with currently available mass spectrometers. Thus, seven ensembles of 64 probes can be used (a total of 448 probes). Results obtained in this embodiment are presented in Figures 5 and 6.

The synthesis of peptides on an automated synthesizer takes place from the C-terminal end to the N-terminal end, the synthesis of nucleic acids from the 3'- to the 5'-end. It is possible to attach a primary aminofunction to one or two end(s) in order to achieve a shift of mass by one or two functionalizations. Alternatively, the mass shifting of a library of combinatorially produced probes can be achieved by applying some building blocks of defined mass (e.g. amino acids in a combinatorial synthesis of PNAs) before the combinatorial building blocks are built in. The first combinatorial synthesis starts directly at the support. For the second combinatorial synthesis first e.g. two valines are coupled. Valine has a mass of 99 Da. By using two valines it is possible to achieve a change in mass from the second combinatorial synthesis to the first by 198 Da. During the third combinatorial synthesis initially four valines are coupled which means that the mass of this ensemble is higher by 396 Da and so on. A possibly necessary charge tag can still be attached at the N-terminal end afterwards with the method described above. Another possibility is to first couple the

charge tag with the solid phase and subsequently continue with the combinatorial synthesis.

Another alternative is to attach several fixed charges of the same polarity simultaneously so that the detection range can be reduced. In a mass spectrometer a molecule with two charges is observed with the corresponding half of the mass. This can be an advantage if the resolution of the mass spectrometers decreases drastically towards higher masses. It was mentioned earlier how to apply positively fixed charges to a probe. Negatively fixed charges can have similar advantages.

Positively and negatively fixed charges can be produced with similar methods.

The descriptions made in connection with the mass tags apply accordingly for the localization, number and combinatorics of the charge tags. Thus, the sensitivity of the analysis can be improved by charge tagging at the 5' or 3'-terminal of the library of nucleic acid probes. This particularly applies when by the introduction of alkylphophonate groups at the non-randomized positions (cf. below) a neutral charge would be created otherwise. The mass tagging which is inevitably connected with the charge tagging facilitates the analysis of the fragments resulting from the cleavage at the phophorothioate groups. Phosphoroselenoates can also be used in order to obtain a preferred cleavage at certain positions.

In another preferred embodiment of the method of the invention the probes are modified nucleic acid molecules.

In a particularly preferred embodiment these modified nucleic acid molecules are PNAs, alkyliated phosphorothioate nucleic acids or alkylphosphonate nucleic acids.

In another preferred embodiment the probes of the method of the invention are produced by means of combinatorial solid phase synthesis. This takes place e.g. via common Boc solid phase synthesis which is also used commercially for the synthesis of PNA pure substances. There, all four or more than one of the bases adenine, guanine, cytosine or thymine are inserted side by side in selected positions within an otherwise fixed sequence of the probe-PNA. This takes place by using not only one but several synthesis building blocks in a step during the solid phase synthesis. By means of variations in several such positions a PNA library is created. Subsequent to the PNA synthesis charge tagging can be carried out at the free terminal aminofunction. This improves the dynamic area of the PNA-analysis by means of MALDI.

In a particularly preferred embodiment the various building blocks are thus marked in the solid phase synthesis in such a way that their masses or the masses of the probes which have been synthesized by them can be differentiated in the mass spectrometer. The marking is accomplished by introducing a different mass modification at the backbone in a way corresponding to the base (Figure 7). In this way the synthesized probes get masses specific to their sequences. If probes bind to a target DNA which has been immobilized at the MALDI-target the accessible mass information in the MALDI experiment allow for unequivocal conclusions to be drawn with regard to the sequences of the hybridized PNA probes.

In another particularly preferred embodiment of the method of the invention the base building blocks are marked with a methyl-, ethyl-, propyl-, a branched or non-branched alkyl-, a halogen substituted branched or non-branched alkyl-, alkoxyalkyl-, alkylaryl, arylakyl-, alkoxyaryl- or aryloxyalkyl-group or with their deuterated or otherwise isotopic variants. Since by using unmodified building blocks the molecular mass allows for conclusions with regard to the base composition at the most but not with regard to the sequence, mass labelled building blocks (i.e. PNA monomers substituted at the backbone) are used which are characteristic to any randomized position within the PNA library. Since in this way the synthesis building block of a certain base in position x has another mass than the one in position y, even different sequences with the same gross composition of the bases can be differentiated due to the molecular mass. The corresponding substituents are selected by numeric value so that any possible sequence corresponds to a fixed mass unequivocally due to the described nature of the library synthesis.

The synthesis of nucleic acid probe libraries takes place at the synthesizer by inserting mixtures of various nucleoside derivates (usually phophoramidites) at the positions to be randomized. The libraries resulting therefrom can also be used as probes in the above-mentioned method. A specific linkage breaking should be carried out e.g. at the fixed phophodiester links which are located at a fixed side of the randomized positions in order to differentiate the different sequences.

In another preferred embodiment of the method of the invention the probes have at least one modification in a defined position away from randomized nucleotides which allow for a cleavage of the probe.

In a particularly preferred embodiment this modification is an introduction of a phophorothioate group and/or a RNA base and/or a phosphotriester bond.

If a probe has three randomized positions, at least two such linkage breakings are necessary (Figure 10). Then, three fragments are produced which contain a randomized position each and which therefore, due to the otherwise known composition of their mass, allow for direct conclusions regarding the variable base. The cleavage at the described linkings can take place in an incomplete manner. This allows for the integration of bigger fragments to secure the sequence information or, in case of ambiguities, to concretize the sequence information. The specific linkage breaking at the randomized positions is carried out by inserting phosphorothioate groups there already during the synthesis of the library. Firstly, these can be hydroxyalkylated with hydroxyalkylhalides and then they can be cleaved selectively under alkaline conditions. Alternatively, sample can be constructed in such a way that uracil is inserted next to a randomized position. By means of uracil-DNA glycosylase and subsequent alkaline treatment the backbone can then be broken at this position.

Usually the matrix is chosen is such a way that it exhibits a high extinction coefficient as well as a good support of the charge formation at the chosen laser wave length. In another preferred embodiment of the method of the invention the matrix supporting the desorption of the probes by means of the laser beam consists of a solution of α-cyano-4-hydroxy cinnamic acid in acetone at a ratio of 1:9 to 9:1, preferably at a ratio of 1:1, or a mixture of α-cyano-4-hydroxy cinnamic acid methyl ester and α-cyano-4-methoxy cinnamic acid or sinapic acid or its methyl derivates at a ratio of 1:9 to 9:1, preferably at a ratio of 1:1.

In another preferred embodiment of the method of the invention the matrix consists of α-cyano-4-hydroxy cinnamic acid or a mixture of α-cyano-4-hydroxy cinnamic acid methyl ester and a α-cyano-4-methoxy cinnamic acid or α-cyano-4-hydroxy cinnamic acid or sinapic acid or its methyl derivate at a ratio of 1:99 to 99:1, preferably at a ratio of 1:1, which is applied to the MALDI probe support as a solution in acetone, isopropanol, acetonitrol, ethanol, methanol or water or in a mixture of two or more of these solvents.

The above-described principle of mass tagging for certain building blocks in fixed positions can be used for various partial libraries which can then be united to form a bigger library.

Thus, in another preferred embodiment the probes are produced as partial libraries having different mass and/or charge tags. It is necessary to mass label also the partial libraries in their syntheses so that specific analysed masses allow for conclusions as to that specific partial library. This takes place purposefully through natural amino acids which can easily be bound to the PNA library in a solid phase synthesis. By mass

labelling various partial libraries the mass range which is at disposal for the analysis of the whole library can be increased significantly.

Furthermore, the present invention relates to a kit containing the probes and/or a probe support, possibly with the bound nucleic acid molecules. The protein support is, as presented above, pretreated in its surface and thus allows for the linking of the nucleic acids. Preferably, the pretreatment takes place in a chemical manner.

A number of publications have been mentioned in this application which hereby have been incorporated into the application by reference.

The figures show:

Figure 1

Scheme of the fingerprinting with mass spectrometric scanning.

1) Combinatorially produced library of probes which can be differentiated in their mass.
2) The library is hybridized to a target DNA which is immobilized on a MALDI target. Only probes with complementary sequences to the sequences in the target DNA settle on the target DNA. After intense washing is MALDI matrix is applied.
3) Hybridized probes are identified by mass analysis.

Figure 2

Schematic illustration of the coating of a MALDI target and immobilization of an array of target DNAs. With non-covalent immobilization the bifunctional linker is left out.

Figure 3

N-terminal mass/charge tagging.

A charge tag can be inserted at the 5' of a nucleic acid or a modified version of a nucleic acid or at the N-terminal end of a PNA. The charge tag carries a N-hydroxy-succinimidester function and a quaternary ammonium group. These two functionalizations are separated by a group $R_1$. $R_1$ serves the mass variation of the charge tags (the coupling of the charge tags is carried out at slightly alkaline pH (8.5) in aqueous solution on ice. The reaction is completed within 30 minutes).

Figure 4

1:1 mixture of unmodified PNA and charge tag PNA.

A charge tag and unmodified PNA have been analysed in a 1:1 mixture with a α-cyano-4-hydroxy cinnamic acid methyl ester matrix. The different sensitivity is obvious. The charge

tagged error sequence (1983 Da) which is shortened by one base gives a significantly stronger signal than the unmodified sequence (2074 Da).

Figure 5

Mass distribution of a 10-mer library.

Two positions in the sequence are synthesized with degenerated bases (TCGA). The sequence is NTTGTTTTCN. Thus, the result are 9 PNAs which can be differentiated in their mass. In the PNA library CC = 2810 Da, CT = TC = 2825 Da, CA = AC = 2834 Da, TT = 2840 Da, TA = AT = 2849 Da which can not be differentiated from CG = GC = 2850 Da, AA = 2858 Da, TG = GT = 2865 Da, AG = GA = 2874 Da and GG = 2890 Da. An isotopic resolution is observed all the way through. The whole PNA library received a charge tag in a "hot pot" reaction. The MALDI analysis was carried out in a 1:1 matrix mixture of α-cyano-4 methoxy cinnamic acid and α-cyano-4-hydroxy cinnamic acid methyl ester.

Figure 6

Mass/charge tagging.

A 10-mer PNA library with 2 degenerately synthesized positions were labelled with a mass/charge tag. The sequence is NTTGTTTTCN. A mixture of starting material and labelled PNA library in a α-cyano-4-hydroxy cinnamic acid matrix is shown. The advantage of applying a fix charge carrier to the library is obvious. The signals form the charge tagged library are detectable with equal concentrations whereas the unmodified PNAs are undetectable. The error sequence which is shortened by one base can be detected.

Figure 7

Principle of the synthesis of PNA libraries by using mass labelled syntheses as well as mass labelled building blocks L (randomized position) carried out simultaneously. In the randomized positions various bases with different substituents at the PNA backbone each (as mass tags) are used. The mass of the corresponding PNA molecule is clearly assigned to its sequence.

Figure 8

Calculated MALDI mass spectrum of a PNA library.

Two different solid phase syntheses (one of them is mass labelled) with 32 different sequences each form 64 different mass peaks each of which is assigned to a specific sequence from a PNA library with 3 variable positions with four bases (A, C, G and T are inserted in each). The calculations are based on the substituents illustrated in Table 1. The computer program MASP (© Dr. Christoph Steinbeck) was used for the calculations.

Figure 9

Possible PNA synthesis building blocks for the combinatorial Boc solid phase synthesis as used for the synthesis of PNA libraries which can be differentiated in their mass. The mass labelled building blocks presented in Table 1 are illustrated.

Figure 10

Sequence analysis of probes by means of specific linking breakings.

The various sequences of a library of DNA probes can be identified mass spectrometrically also through specific linking breakings at the randomized positions (N = A, G, C or T). Already during the solid phase synthesis a phosphorothioate function is inserted at these positions where the DNA can be cleaved specifically (e.g. with iodoehtanol). This is schematically illustrated without considering the exact chemical nature of the fragments. Since their sequence is mainly known, the mass of the fragments allows for an exact determination of the whole sequence without complete sequencing having to take place. The substituent $R_1$ denotes any other DNA sequence, R2 another sequence or –H. R3 denotes –OH (phosphodiester) or alkyl- (alkylphophonate).

Figure 11

Charge tagged PNA probe mixture CCXCAGCC (X = A, G, C, T) hybridizes to a target DNA immobilized on eupergit, CCCCAGCC is the complementary sequence. The lower spectrum shows the starting mixture, the higher spectrum a clearly preferred hybridization of the complementary CCCCAGCC sequence.

Figure 12

Charge tagged CCTCAGCC complementary to the target DNA immobilzed on the MALDI probe support, detected after application of the 4-hydroxy-$\alpha$-cyano cinnamic acid matrix without movement of the probe support during measurement.

The examples illustrate the invention.

Example 1: Description of the whole method of the invention

A MALDI target is coated with a substance which allows for the immobilization of a chemically modified PCR products (e.g. a $NH_2$ functionalized surface). An array of target DNAs is transferred to that surface and bound. The array is hybridized with a library of probes produced in the described manner. Complementary components of the probe library bind to the target DNAs. They are subsequently washed thoroughly to remove

non-bound probes. The matrix is applied and the MALDI target is transferred to the mass spectrometer. Each point where there is a target DNA is aimed at with the laser and a mass analysis of the probes bound to that target DNA is carried out.

Example 2: Analysis of a charge tagged PNA library

A library of PNAs is charge tagged in a synthesis as described herein. The library of PNA is dissolved in 50 % acetonitril and diluted. The MALDI matrix (in this case a 1:1 mixture of α-cyano-4-methoxy cinnamic acid and α-cyano-4-hydroxy cinnamic acid methyl ester in acetone) is applied to the MALDI target. The solvent evaporates immediately. Then, 0.5 ml of the CT-PNA solution is applied to the dried matrix. After evaporation of the solvent the MALDI target is transferred into the mass spectrometer and the probe is analysed. The result is shown in Figure 5.

Example 3: Analysis of an array of target DNAs through CT-PNAs

A library of CT-PNAs produced in the combinatorial way described is hybridized to an array of target DNAs which is immobilized on a MALDI target. The probes which find a complementary sequence in a target DNA are bound. Then, the MALDI target is washed to remove all remaining components of the library. The matrix is applied to the MALDI target and the target is transferred to the mass spectrometer for analysis (Figure 1). An unknown target DNA is characterized by the hybridized probes. Similarities between various target DNAs are characterized by the fact that they bind the same probes.

Example 4: Analysis of an array of target DNAs by means of probes

Probes are produced individually and subsequently united to form a library. It is essential that no mass is occupied by two different probes. This library of probes is used for hybridization to an array of immobilized target DNAs. The non-bound probes are washed off. The matrix is applied and the bound probes are analysed in the mass spectrometer.

Example 5: Production of partial libraries and MALDI analysis

A preferred example of a PNA library with a clear relation between the mass and the sequence is shown in Figure 8 and Table 1. In an otherwise random PNA three positions should be varied in the combinatorial solid phase synthesis. This corresponds to 64 possible compounds with 4 different bases. In the present example two separate syntheses are carried out with the components given in Table 1. Synthesis 2 is additionally mass labelled by adding of two valine units. Each partial synthesis provides

32 compounds which can be differentiated by their mass. Both partial libraries can be united to form a library with 64 different sequences with a specific mass of each. The calculated MALDI mass spectrum for this library is shown in Figure 8. The 64 peaks do not overlap; each peak corresponds to a specific sequence form a PNA library with three randomized base positions (4 bases each).

Example 6: Surface treatment of the MALDI probe support

In a preferred variation of the method the surface of the MALDI probe support is first coated with silicate and then epoxy-functionalized by means of silinisation. Alternatively, an epoxy-functionalized acryl polymer can also be applied to the metal surface. The target DNA is bound covalently to the surface by means of the epoxy functions. In a particularly preferred variation the DNA to be immobilized is first supplied with a primary amino function. The epoxy functions which have not reacted are subsequently deactivated with a surplus of an amine and the method is then continued according to example 4.

Example 7: Construction of a probe library of 448 probe.

7 syntheses are carried out according to the method described in the last example and subsequently the mass/charge tags are coupled.

| | mass range |
|---|---|
| 1. 64 probes: charge tag- $TCP_1GAP_2GAP_3G$ | 2600-2800 Da |
| 2. 64 probes: charge tag+200 Da mass tag- $TCP_1AGP_2GAP_3G$ | 2800-3000 Da |
| 3. 64 probes: charge tag+400 Da mass tag- $TCP_1AGP_2AGP_3G$ | 3000-3200 Da |
| 4. 64 probes: charge tag+600 Da mass tag- $TCP_1AAP_2AGP_3G$ | 3200-3400 Da |
| 5. 64 probes: charge tag+800 Da mass tag- $TCP_1AAP_2GAP_3G$ | 3400-3600 Da |
| 6. 64 probes: charge tag+1000 Da mass tag- $TCP_1GAP2GAP_3G$ | 3600-3800 Da |
| 7. 64 probes: charge tag+1200 Da mass tag- $TCP_1GAP_2AGP_3G$ | 3800-4000 Da |

In the above-mentioned synthesis series, the sixth synthesis serves as internal control. Alternatively, the following synthesis can also be carried out:

6. 64 probes: charge tag+ 1000 Da mass tag- $TCP_1GGP_2GAP_3G$ 3600-3800 Da.

Example 8: Covalent immobilization of a target DNA on a metal surface.

1. Coating of the metal surface with silicate and silanes:

Variant A:

50 mg sodiumtrisilicate (Aldrich) is dissolved in 600 µl water under heating and stirring and 100 µl methanol is added in drops. While standing the solution must not become turbid within 15 minutes. An even coating of this solution is applied to the pre-cleaned plate with a soft cloth and dried at room temperature. The plate is further dried for 15 min at 50 °C and washed in methanol. Then it is reheated for 15 min at 50°C and unpolymerized silicate is washed down with water. The "glassy" metal plate is treated for 20 min in a solution of 3-(aminopropyl)-triethoxysilane (2% in acetone:water/95:5), washed with methanol and dried at 50°C.

Variant B: (preferred variant)

As described in A) 5% 3-(aminopropyl)-triethoxysilane is added to the silicate solution and an even layer thereof is applied onto the metal plate with a soft cloth. After drying at room temperature, the surface is subjected to the vapours of concentrated hydrochloric acid for 30 s which causes it to cloud opaque. The plate is dried for 15 min at 50°C, washed with methanol and water and subsequently dried again.

Variant C:

The pre-cleaned metal plate is silanisated with a solution of 3-(aminopropyl)-triethoxysilane (2% in acetone:water/95:5) for 30 min. The plate is dried, washed with methanol, heated to a temperature of 50°C for 15 min and washed with water.

Variant D:

Analogous to the variants A, B and C the functionalization with thiols by using 3-(mercaptopropyl)-triethoxysilane is possible.

2. Pretreatment of a metal carrier with epoxysilanes:

A metal probe support is coated with silicate as described above. The surface is washed with bidistilled water several times. The dried probe support is coated with a 1:1 mixture of 3-glycidyloxypropyl-trimethoxysilane and trimethoxy[2-(7-oxabicyclo[4.1.0]hept-3-yl)ethyl]-silane (0.5µl/$mm^2$) and incubated on the heating plate for 40 min. The plates are washed thoroughly with acetone, dried at 55°C for 15 min. and stored in vacuum.

3. Functionalization of DNA with a linker:

Variant A:
DNA synthesized with a phosphorothioate bridge is functionalized with a surplus SIAB (4-(iodacetamido)-benzoeacid N-hydroxysuccinimidylester, Pierce Chemical, Rockford, IL USA) in DMF. The solvent is steamed off in vacuum, the yellow residue washed several times with ethyl acetate and then dried. Under these conditions the iodacetamido function reacts exclusively. The N-hydroxy-succinimidester remains available for the subsequent reaction with amino functions in the course of the immobilization.

Variant B:
The amino-functionalized oligonucleotide is caused to react with a surplus of SIAB in anhydrous DMSO at room temperature. The purification is carried out as under A). Under these conditions SIAB reacts at about 50% with its iodoacetamido function and at about 50% with N-hydroxy-succinimidester function. The immobilization can then be carried out in reverse at a metal plate which has been treated according to 1D.

Variant C:
The variants A) and B) can also be carried out analogously with halogenalkylcarboxylic-acid-NHS-esters. Long reaction times and increased temperature, however, are necessary.

4. Binding of DNA on pretreated metal plates:

Variant A:
The functionalized DNS is dissolved in a saturated solution of sodium acetate in anhydrous DMSO and applied onto the coated metal plate. After 30 min reaction time the remaining solvent is removed and the metal plate is washed first with 1 M ammoniumchloride solution and then with bidistilled water in such a way that a mutual contamination of the neighbouring spots can be avoided. This process is repeated three times. Then a lot of bidistilled water is used for rinsing again and the metal plate is stored in vacuum until hybridization takes places subsequently.

Variant B:
Analogous to A) also the non-covalent immobilization with unmodified DNA is possible. In this case the washing process takes place without the 1 M ammoniumchloride solution.

Varinat C:

The immobilization of target DNA on MALDI targets can also be carried out in the following manner. The amino-functionalized DNA (1.5 nmol) is added to immobilization buffer (1 M $K_2HPO_4/KH_2PO_4$ pH 7.5). The surfaces which have been functionalized by silanisation are coated with this solution and left to stand for 16 to 20 hours at room temperature. The plates are subsequently washed with hybridization buffer several times and dried.

Variant D:

The immobilization of target DNA on a epoxy-functionalized solid phase can be carried out as follows. 10 mg support (Eupergit C250 L, Röhm Pharma Polymere) are suspended in 1 ml immobilization buffer (1M $K_2HPO_4/KH_2PO_4$ pH 7.5). 1.5 nmol are added to the target DNA to be immobilized and incubated for 24 hours at room temperature while stirring gently. The surplus is removed and the epoxy functions which have not been caused to react are deactivated by treating them with 1 M glycine solution at room temperature. The residue is removed, the support is washed several times. In that way the immobilized DNA can be stored at –20°C for some time.

5. Hybridization and preparation of samples:

The hybridization on the immobilized target DNA with PNA probes (or nucleic acid probes) takes place in PBS buffer at a temperature adjusted to the corresponding probe. The target is washed first with PBS buffer, then again with bidistilled water. The metal plate is dried in vacuum and subsequently coated with the MALDI matrix (α-cyano-4-hydroxy cinnamic acid, 1% in acetone; or analogous to the corresponding methyl esters for the method with charge tagged PNAs/nucleic acids or, as a preferred variant, a 1:1 mixture of the α-cyano-4-hydroxy cinnamic acid methyl ester with α-cyano-4-methoxy cinnamic acid for the charge tagged PNA for each spot separately. Alternatively flat coating with a matrix spray can also be carried out. Due to the fast drying process a diffusion within the array is avoided.

5. Preferred variant

In the preferred variant the linking of a DNA which has been synthesized with a phophorothioate bridge and functionalized with SIAB according to 2.A onto a surface produced according to 1.B on a metal plate by means of reaction in anhydrous DMSO at room temperature (30 min). After hybridization with the charge tagged probe PNA/DNA the MALDI target is washed with bidistilled water, dried and for each spot separated flat

coated with a mixture of α-cyano-4-hydroxy cinnamic acid methyl ester and α-cyano-4-methoxy cinnamic acid (as under 4.).

6. Protein coating:

Another variant is the coating of the metal surface of the MALDI target with protein. Gene32, a protein binding single DNA in a sequence-unspecific way, is suitable for that. After coating the target with this protein an array of the target DNAs can be applied to it. If the array of the target DNA is of cDNA, these have been primed with oligo-dT (e.g. dTTTTTTTTTTTTT) in the PCR. Oligo-dT interacts strongly with Gene32. The covalent linkage of the oligo-dT to Gene32 can be achieved by means of photo crosslinking with short UV light. [36,37] After the immobilization of the MALDI target a library of probes can be used in the described manner to analyse the array of the target DNA. Sequence-specific protein/DNA interactions, such as e.g. GCN4/AP1 are suitable, too.

Another variant is to bind biotinylated target DNA to a magnetic bead coated with streptavidin. This target DNA is analysed with a probe library and the magnetic particles are subsequently transferred to the MALDI target where the probes are transferred to the maxtrix by the matrix and light heating.

| Base | Position 1 | Position 2 | Position 3 |
|---|---|---|---|
| A | H | *i*Pr | H |
| T | H | Me | *i*PrOCH$_2$ |
| C | H | H | *i*PrOCH$_2$ * |
| G | H | *i*Bu | H * |

Table 1: Substituentes applied to the PNA subunits for the production of a mass labelled library with clear mass/sequence relation. *: second synthesis mass labelled with 2 valine units. The corresponding synthesis components are shown in Fig. 9.

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